## REMARKS

Applicants have canceled Claim 53, without prejudice to, or disclaimer of, the subject matter contained therein, and have added new Claims 60-72. Applicants maintain that the cancellation of a claim makes no admission as to its patentability and reserve the right to pursue the subject matter of the cancelled claims in this or any other patent application.

Applicants have amended Claims 40-47, and 50-52 to delete reference to the figures.

Applicants submit that no new matter has been added by the amendments, and that support for the amendments can be found throughout the specification. Support for Claim 60 can be found, for example, at page 82, line 1 and throughout the specification. Support for new Claims 61-71 can be found, for example, in the claims as originally filed, and page 82, lines 21-28.

Claims 40-47, 50-54, and 56-71 are presented for further examination. Applicants respond below to the specific rejections raised by the Examiner in the Office Action mailed March 16, 2005. For the reasons set forth below, Applicants respectfully traverse.

**Rejection Under 35 U.S.C. § 101 – Utility:**

The Examiner has maintained the rejection of Claims 40-47, 50-53, and 56-59 as lacking a specific, substantial, and credible utility for the reasons set forth in the Office Actions mailed April 28, 2004 and October 28, 2004. In particular, the Examiner states that the asserted utilities for the claimed nucleic acids set forth in the specification and Office Action responses – grounded on the utility of PRO444 as a diagnostic marker for pericyte-associated tumors, use as a reagent to identify and isolate PRO444 antagonists, and use as a therapeutic to stimulate angiogenesis - are unpersuasive. The Examiner alleges that there is no evidence that PRO444 is exclusively present or expressed at altered levels in pericyte-associated tumors, and hence PRO444 cannot be used as a marker for the same. The Examiner also argues that there is no evidence that PRO444-induced activation of *c-fos* is specifically related to pericyte-associated tumors or angiogenesis.

The Examiner maintains that Dr. Gerritsen's Declaration is insufficient to overcome the rejection. Specifically, the Examiner asserts that Dr. Gerritsen's declaration is not backed by scientific literature showing the importance of pericytes in regulating angiogenesis or the role of

*c-fos* in regulating cancer and angiogenesis. The Examiner maintains that, as Dr. Gerristen testifies, many growth factors are known to induce *c-fos*. The Examiner cites to Sakurai et al. and Otani et al. as supporting evidence of the various known regulators of *c-fos*. The Examiner concludes that since PRO444 is not the only regulator of *c-fos* there is thus "no specific biological function that could be particularly attributed to PRO444 with respect to its ability to activate *c-fos* expression in pericytes." Office Action at 5. The Examiner also cites to Ozerdem et al. for the proposition that the role of pericytes in the formation of tumor neovasculature varies depending on the type of tissue and tumor, and concludes that PRO444 cannot be specifically associated with the onset of cancer and angiogenesis.

Applicants respectfully disagree.

*Utility – Legal Standard*

According to the Utility Examination Guidelines ("Utility Guidelines"), 66 Fed. Reg. 1092 (2001) an invention complies with the utility requirement of 35 U.S.C. § 101, if it has at least one asserted "specific, substantial, and credible utility" or a "well-established utility."

Under the Utility Guidelines, a utility is "specific" when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic tool without also identifying the condition that is to be diagnosed. By contrast, the utility requirement is satisfied when "an applicant discloses a specific biological activity and reasonably correlates that activity to a disease condition." M.P.E.P. § 2107.01(I).

The requirement of "substantial utility" defines a "real world" use, and derives from the Supreme Court's holding in *Brenner v. Manson*, 383 U.S. 519, 534 (1966) stating that "The basic *quid pro quo* contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility." In explaining the "substantial utility" standard, M.P.E.P. § 2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. "Rather, *any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient,* at least with regard to defining a 'substantial' utility." (M.P.E.P. § 2107.01, emphasis added).

Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement, set forth in M.P.E.P. § 2107 II(B)(1) gives the following instruction to patent examiners: "If the applicant has asserted that the claimed invention is useful for any particular practical purpose ... and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility."

*Utility need NOT be Proved to a Statistical Certainty – a Reasonable Correlation between the Evidence and the Asserted Utility is Sufficient*

An Applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. § 101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope." *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA 1974). *See, also In re Jolles*, 628 F.2d 1322, 206 USPQ 885 (CCPA 1980); *In re Irons*, 340 F.2d 974, 144 USPQ 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 USPQ 209, 212-13 (CCPA 1977). Compliance with 35 U.S.C. § 101 is a question of fact. *Raytheon v. Roper*, 724 F.2d 951, 956, 220 USPQ 592, 596 (Fed. Cir. 1983) cert. denied, 469 US 835 (1984). The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the evidence, or "more likely than not" standard. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). This is stated explicitly in the M.P.E.P.:

> [T]he applicant does not have to provide evidence sufficient to establish that an asserted utility is true "beyond a reasonable doubt." **Nor must the applicant provide evidence such that it establishes an asserted utility as a matter of statistical certainty**. Instead, evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true. M.P.E.P. at § 2107.02, part VII (2004) (underline emphasis in original, bold emphasis added, internal citations omitted).

The PTO has the initial burden to offer evidence "that one of ordinary skill in the art would reasonably doubt the asserted utility." *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). Only then does the burden shift to the Applicant to provide rebuttal evidence. *Id.* As stated in the M.P.E.P., such rebuttal evidence does not need to absolutely prove that the asserted utility is real. Rather, the evidence only needs to be reasonably indicative of the asserted utility.

In *Fujikawa v. Wattanasin*, 93 F.3d 1559, 39 U.S.P.Q.2d 1895 (Fed. Cir. 1996), the Court of Appeals for the Federal Circuit upheld a PTO decision that *in vitro* testing of a novel pharmaceutical compound was sufficient to establish practical utility, stating the following rule:

> [T]esting is often required to establish practical utility. But the test results **need not absolutely prove** that the compound is pharmacologically active. All that is required is that the tests be "*reasonably* indicative of the desired [pharmacological] response." In other words, there must be **a sufficient correlation** between the tests and an asserted pharmacological activity so as to convince those skilled in the art, **to a reasonable probability**, that the novel compound will exhibit the asserted pharmacological behavior." *Fujikawa v. Wattanasin*, 93 F.3d 1559, 1564, 39 U.S.P.Q.2d 1895 (Fed. Cir. 1996) (internal citations omitted, bold emphasis added, italics in original).

While the *Fujikawa* case was in the context of utility for pharmaceutical compounds, the principals stated by the Court are applicable in the instant case where the asserted utility is for a diagnostic use – utility does not have to be established to an absolute certainty, rather, the evidence must convince a person of skill in the art "to a reasonable probability." In addition, the evidence need not be direct, so long as there is a "sufficient correlation" between the tests performed and the asserted utility.

Thus, the legal standard for demonstrating utility is a relatively low hurdle. An Applicant need only provide evidence such that it is **more likely than not that a person of skill in the art would be convinced, to a reasonable probability, that the asserted utility is true**. The evidence need not be direct evidence, so long as there is a reasonable correlation between the evidence and the asserted utility. The Applicant **does not need to provide evidence such that it establishes an asserted utility as a matter of statistical certainty.**

Even assuming that the PTO has met its initial burden to offer evidence that one of ordinary skill in the art would reasonably doubt the truth of the asserted utility, Applicants assert that they have met their burden of providing rebuttal evidence such that it is more likely than not those skilled in the art, to a reasonable probability, would believe that the claimed invention is useful for the diagnosis and treatment of pericyte-associated tumors and for the stimulation of angiogenesis.

In an attempt to clarify Applicants' argument, Applicants offer a summary of their argument and the disputes issues involved. Applicants assert they have provided reliable evidence that PRO444 stimulates *c-fos* in pericytes. Given the established role of pericytes in

regulating angiogenesis, and the established role of *c-fos* in cancer and in stimulating pericytes, Applicants submit that the claimed nucleic acids are useful in generating therapeutics in treating pericyte-associated tumors as well as stimulating angiogenesis.

Applicants understand the Examiner to be making the following arguments in response to Applicants' asserted utilities:

1. The Examiner argues that there is no evidence that *c-fos* induction is associated with cancer or angiogenesis. Office Action at 4-5.

2. Citing Otani et al. Sakurai et al. and Coulon et al., the Examiner argues that several growth factors are capable of inducing *c-fos* expression, and therefore PRO444-induced *c-fos* induction is not a biological activity that can be particularly attributed to PRO444.

3. Citing Ozerdem et al., the Examiner argues that the role of pericytes in angiogenesis is not fully understood, and therefore induction of *c-fos* cannot be specifically associated with cancer or angiogenesis.

4. The Examiner argues that there is no evidence that PRO444 is present exclusively or expressed at altered levels in pericyte-associated tumors.

As detailed below, Applicants submit that the PTO has failed to meet its initial burden to offer evidence "that one of ordinary skill in the art would reasonably doubt the asserted utility." *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). The Examiner has failed to offer evidence to support the rejection of the evidence provided in the form of scientific articles and the Gerritsen declaration (i.e., that *c-fos* induction is associated with cancer or angiogenesis). To the contrary, analysis of the evidence relied upon by the Examiner establishes the association between *c-fos* induction in pericytes and angiogenesis. The fact that other known regulators of *c-fos* expression exist is irrelevant regarding whether PRO444 is a specific regulator of *c-fos*.

Applicants address each of the Examiner's arguments in turn.

*Applicants have provided evidence that c-fos induction is associated with cancer and angiogenesis*

Applicants submit that the scientific literature illustrates the well-established role of *c-fos* induction in cancer and angiogenesis. For example, in Applicants' Office Action Response mailed July 27, 2004, Applicants referenced Saez et al., a study involving *c-fos* knockout mice

that demonstrated the requirement of *c-fos* for the development of malignant tumors. Saez emphasizes that their data "highlight the significance of *c-fos* for full neoplastic development." Saez, at 730. Applicants also previously submitted a study by Marconcini et al., which establishes the importance of *c-fos* in angiogenesis. The Marconcini study demonstrated the central role of VEGF-D, a growth factor known to be induced by *c-fos*, in angiogenesis. The authors note that angiogenic activity arising from *c-fos* mediated induction of VEGF "strongly suggests that VEGF-D can be a *c-fos* effector for tumor malignancy." Marconcini et al., at 9676.

In further support of Applicants' position, a review article by McColl et al. is submitted herewith as Exhibit 1. McColl discusses the VEGF family of proteins that are "central to angiogenesis." McColl et al., at 463. McColl notes that "**the association between increased transcription [of VEGF-D –a regulator of angiogenesis] mediated by [*c-fos*] and tumorigenesis is well established.**" (McColl et al. at 471, emphasis added, internal citations omitted). In light of the above, Applicants submit that they have provided sufficient evidence of a specific activity (*c-fos* induction in pericytes) and have reasonably correlated this activity with a disease condition (tumorigenesis). These references clearly establish that one skilled in the art would believe that it is more likely than not that PRO444, an inducer of *c-fos* activity in cells having a known role in angiogenesis, (*i.e,* pericytes), is useful as an angiogenic factor. Further support is found in the three references cited by the Examiner, as discussed below.

*The Arguments made by the Examiner are not Sufficient to Satisfy the PTO's Initial Burden of Offering Evidence "that one of ordinary skill in the art would reasonably doubt the asserted utility"*

As stated above, an Applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. § 101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope." *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA 1974). The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the evidence, or "more likely than not" standard. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). This is stated explicitly in the M.P.E.P.:

> [T]he applicant does not have to provide evidence sufficient to establish that an asserted utility is true "beyond a reasonable doubt." **Nor must the applicant provide evidence such that it establishes an asserted utility as a matter of**

> **statistical certainty**. Instead, evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true. M.P.E.P. at § 2107.02, part VII (2004) (underline emphasis in original, bold emphasis added, internal citations omitted).

The PTO has the initial burden to offer evidence "that one of ordinary skill in the art would reasonably doubt the asserted utility." *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). Only then does the burden shift to the Applicant to provide rebuttal evidence. *Id.* As stated in the M.P.E.P., such rebuttal evidence does not need to absolutely prove that the asserted utility is real. Rather, the evidence only needs to be reasonably indicative of the asserted utility.

Considering the totality of the evidence of record, Applicants submit that the PTO has not met its initial burden of overcoming the presumption that the asserted utility is sufficient to satisfy the utility requirement. The evidence proffered by the Examiner to counter Applicants' own evidence not only fails to cast doubt on Applicants' asserted utilities, but it demonstrates the role of *c-fos* and pericytes in angiogenesis and neovasculature. Applicants address each reference in turn.

According to the Examiner, Otani et al. teaches *c-fos* activation in pericytes by angiotensin II and VEGF. The Examiner maintains that this evinces that there is "no specific biological function that could be particularly attributed to PRO444 with respect to its ability to activate *c-fos* expression in pericytes." Office Action at 5. As an initial matter, Applicants point out that Otani et al. teach induction of VEGF – "a potent angiogenic factor" (Otani et al., at 1192) – in retinal pericytes by *c-fos*, not induction of *c-fos* by VEGF.

Otani et al. analyzed the effect of Angiotensin II on VEGF expression in pericytes. The authors found that induction of *c-fos* in retinal pericytes (through the angiotensin II pathway) resulted in an increase in VEGF expression. Based on their results, the authors conclude that *c-fos* plays "a predominant role" in VEGF activation in pericytes. Otani et al., at 1197. As VEGF is characterized as "a potent angiogenic factor," Otani et al. demonstrates the logical correlation between *c-fos* induction in pericytes and angiogenesis.

Contrary to the Examiner's assertion, the fact that Angiotensin II is also an inducer of *c-fos* does not detract from Applicants' asserted utility. Applicants do not assert that PRO444 is the sole regulator of *c-fos* activity, or even that it is the sole regulator of *c-fos* activity in

pericytes. While the Examiner "does not dispute the correctness of [Applicants'] experimental protocol," establishing that PRO444 is a specific inducer of *c-fos*, the Examiner nevertheless maintains that "there appears to be no clear physiological meaning attributed to the activation of *c-fos* by PRO444." Office Action at 6. Applicants submit that given that PRO444 can function as an inducer of *c-fos* in pericytes, it is more likely than not that VEGF will in turn be activated, which is a "potent angiogenic factor."

Similarly, the Examiner has relied on Sakurai et al. for the proposition since other known regulators of *c-fos* exist, PRO444 lacks utility. Sakurai analyzed proliferation of pericytes in response to prostaglandins. The authors found that prostaglandins stimulated *c-fos* expression in pericytes, and that the increased *c-fos* expression correlated with increased VEGF expression. The authors characterize VEGF as "a key growth factor for retinal neovasculatrization" As with Otani, Sakurai et al. illustrates the logical correlation between *c-fos* induction in pericytes and angiogenesis. For the reasons discussed in connection with Otani, Applicants submit that Sakurai et al. establishes that those skilled in the art appreciate the physiological meaning attributed to the action of *c-fos* in pericytes.

The Examiner also maintains that Coulon et al. teaches that induction of *c-fos* represents a generalized, first line of cellular response. Office Action at 5. In the previous Office Action, the Examiner argued that the fact that *c-fos* is activated in several cell types and is not associated with a specific physiological function, indicates that there is " no scientific logic to conclude that the instant PRO444 polypeptides, as inducers of *c-fos* expression, are involved in tumorigenesis, as asserted in the instant specification." Office Action mailed October 28, 2004, at 4. Coulon et al. teaches that calcium acts synergistically with other stimuli to activate *c-fos* transcription in fibroblast cells. As stated above, Applicants assert that the fact that other regulators of *c-fos* exist has no bearing on Applicants' asserted utilities (*e.g.*, as a diagnostic or therapeutic target for pericyte associated tumors, or as an angiogeneic agent). Applicants have provided evidence that PRO444 stimulates *c-fos* in pericyte cells. In fact, the references cited by the Examiner are supporting evidence that those skilled in the art appreciate the connection between *c-fos* induction and angiogenesis in pericytes. Applicants submit that the teachings of Coulon *et al.* would not lead one skilled in the art to doubt that induction of *c-fos* is associated with angiogenesis in pericyte cells.

The Examiner argues that Ozerdem et al. shows that the role of pericytes in formation of tumor neovasculature is not fully understood, and therefore PRO444 lacks utility because it cannot be specifically associated with the onset of cancer or angiogenesis. Applicants respectfully disagree with the Examiner's characterization of Ozerdem. The Examiner cites to page 241, which reports that the relationship between pericytes and endothelial cells varies from tissue to tissue. Applicants submit that this section of Ozerdem is silent regarding the role of pericytes in the formation of neovasculature. Instead, the focus of the cited section of Ozerdem concerns the relationship between two different types of cells, *.i.e.,* pericytes and endothelial cells. On the other hand, the remainder of Ozerdem confirms the association of pericytes and angiogenesis in retinal tissues. Specifically, Ozerdem notes the central role pericytes play in angiogenesis. *Id.* at 426. As a whole, the study supports Applicants' position that pericytes are linked to angiogenesis, and that one skilled in the art would more likely than not believe that induction of *c-fos* (known to have a central role in angiogenesis) in pericytes.

**Conclusion**

The Examiner has asserted several arguments for why there is a lack of a substantial utility: (1) the Examiner alleges that there is no evidence that *c-fos* induction is associated with cancer or angiogenesis; (2) the Examiner argues that several growth factors are capable of inducing *c-fos* expression, and therefore PRO444-induced *c-fos* induction is not a biological activity that can be particularly attributed to PRO444; (3) the Examiner argues that the role of pericytes in angiogenesis is not fully understood, and therefore induction of *c-fos* cannot be specifically associated with cancer or angiogenesis; and (4) The Examiner argues that there is no evidence that PRO444 is present exclusively or expressed at altered levels in pericyte-associated tumors.

For the above reasons, the Examiner has maintained the position that the claimed nucleic acids cannot be used to stimulate angiogenesis, or as a diagnostic marker or therapeutic target for pericyte-associated tumors. Applicants have addressed each of these arguments in turn.

First, the Applicants provided several references concerning the relationship between *c-fos* induction and angiogenesis and tumor neovascularization. Next, Applicants have demonstrated that the fact that PRO444 is not the exclusive *c-fos* regulator does not detract from Applicants' asserted utility. The Examiner has not offered any substantial reasoning or evidence

to the contrary – the Examiner has cited to references that establish the association between *c-fos*, angiogenesis, and pericytes, supporting Applicants' asserted utility.

Given the totality of the evidence provided, Applicants submit that they have established a substantial, specific, and credible utility for the claimed nucleic acids as angiogenic agents. According to the PTO Utility Examination Guidelines (2001), irrefutable proof of a claimed utility is not required. Rather, a specific, substantial, and credible utility requires only a "reasonable" confirmation of a real world context of use. Applicants remind the PTO that:

> A small degree of utility is sufficient . . . The claimed invention must only be capable of performing some beneficial function . . . An invention does not lack utility merely because the particular embodiment disclosed in the patent lacks perfection or performs crudely . . . A commercially successful product is not required . . . Nor is it essential that the invention accomplish all its intended functions . . . or operate under all conditions . . . partial success being sufficient to demonstrate patentable utility . . . In short, **the defense of non-utility cannot be sustained without proof of total incapacity**. If an invention is only partially successful in achieving a useful result, a rejection of the claimed invention as a whole based on a lack of utility is not appropriate. M.P.E.P. at 2107.01 (underline emphasis in original, bold emphasis added, citations omitted).

Applicants submit that they have established that it is more likely than not that one of skill in the art would reasonably accept the utility for the claimed nucleic acids as tools to induce angiogenesis as set forth in the specification. In view of the above, Applicants respectfully request that the PTO reconsider and withdraw the utility rejection under 35 U.S.C. §101.

**Rejection Under 35 U.S.C. § 112, First Paragraph – Enablement**

The Examiner has maintained the rejection of Claims 40-47, 50-53, and 56-69 as not being enabled since the claimed invention is allegedly not supported by either a specific and substantial asserted utility, or a well-established utility.

Applicants submit that in the discussion of the 35 U.S.C. § 101 rejection above, Applicants have established a substantial, specific, and credible utility for the claimed nucleic acids. Applicants therefore request that the Examiner reconsider and withdraw the enablement rejection to the extent that it is based on a lack of utility for the claimed nucleic acids.

**Rejection Under 35 U.S.C. § 112, First Paragraph – Written Description**

The Examiner has maintained the rejection of Claims 40-44 under 35 U.S.C. § 112, first paragraph, as lacking adequate written description. The Examiner asserts that the specification fails to provide written description for the claimed variants of SEQ ID NO:8, and that while a skilled artisan could determine if a polypeptide induces *c-fos* activation in perictyes, this limitation does not satisfy the written description requirement. The Examiner asserts that the statement that a compound is part of the invention and a reference to a potential method of isolating it is not sufficient, and that the compound itself is required.

*The Legal Standard for Written Description*

The well-established test for sufficiency of support under the written description requirement of 35 U.S.C. §112, first paragraph is whether the disclosure "reasonably conveys to artisan that the inventor had possession at that time of the later claimed subject matter." *In re Kaslow*, 707 F.2d 1366, 1375, 2121 USPQ 1089, 1096 (Fed. Cir. 1983); see also *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at1563, 19 USPQ2d at 1116 (Fed. Cir. 1991). The adequacy of written description support is a factual issue and is to be determined on a case-by-case basis. See e.g., *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116 (Fed. Cir. 1991). The factual determination in a written description analysis depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure. *Union Oil v. Atlantic Richfield Co.*, 208 F.3d 989, 996 (Fed. Cir. 2000).

*The Current Invention is Adequately Described*

As noted above, whether the Applicants were in possession of the invention as of the effective filing date of an application is a factual determination, reached by the consideration of a number of factors, including the level of knowledge and skill in the art, and the teaching provided by the specification. The inventor is not required to describe every single detail of his/her invention. An Applicant's disclosure obligation varies according to the art to which the invention pertains. The present invention pertains to the field of recombinant DNA/protein technology. It is well-established that the level of skill in this field is very high since a representative person of skill is generally a Ph.D. scientist with several years of experience. Accordingly, the teaching imparted in the specification must be evaluated through the eyes of a highly skilled artisan as of the date the invention was made.

The subject matter of the rejected claims concerns nucleic acids encoding a polypeptide having 80% to 99% sequence identity to SEQ ID NO:9, with the functional recitation "wherein said isolated nucleic acid encodes a polypeptide having the ability to induce c-fos expression." Other claimed nucleic acids are those which hybridize to the nucleic acid sequence of SEQ ID NO:8, the full-length coding sequence of the nucleic acid sequence of SEQ ID NO:8, the full-length coding sequence of the cDNA deposited under ATCC accession number 203406, or the complements thereof, under the specified stringent conditions. We turn first to the claims which recite specific high stringency hybridization conditions.

In *Enzo Biochem v. Gen-Probe Inc.*, 323 F.3d 956 (Fed. Cir. 2002), the Court held that functional descriptions of genetic material may satisfy the written description requirement. In so holding, the Court gave judicial notice to the USPTO's Manual of Patent Examining Procedure, which provides that the written description requirement may be satisfied when the disclosure provides sufficiently detailed identifying characteristics, such as "complete or partial structure, other physical and/or chemical properties, *functional characteristics when coupled with a known or disclosed correlation between function and structure*, or some combination of such characteristics." *Id.* at 964, quoting 66 Fed. Reg. at 1106 (emphasis in original). In *Enzo*, the Court found describing nucleic acids based on their ability to hybridize to another nucleic acid sequence which was adequately described may be an adequate description of the nucleic acid. This is because the hybridization function of a nucleic acid is dependent on the sequences of the nucleic acid – a disclosed function which is coupled with a known correlation between function and structure. The Court favorably discussed the PTO's example wherein "genus claims to nucleic acids based on their hybridization properties...may be adequately described if they hybridize under highly stringent conditions to known sequences because <u>such conditions dictate that all species within the genus will be structurally similar</u>." *Id.* at 967 (citing *Application of [Written Description] Guidelines*, Example 9) (emphasis added).

Applicants submit that the stringent hybridization conditions specified in the pending claims, alone or in combination with the recited percent sequence identity, result in all species within the genus being structurally similar. As the *Enzo* Court noted, Examples 9 and 10 of the Application of Written Description Guidelines (hereinafter "Guidelines") make clear that specifying hybridization under highly stringent conditions yields "structurally similar DNAs."

Guidelines, Example 9 at page 36. The analysis of a genus claim in Example 10 of the Guidelines states:

> [T]urning to the genus analysis, the art indicates that *there is no substantial variation within the [claimed] genus because of the stringency of hybridization conditions which yields structurally similar molecules.* The single disclosed species is representative of the genus because reduction to practice of this species, considered along with the defined hybridization conditions and the level of skill and knowledge in the art, are sufficient to allow the skilled artisan to recognize that applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus. Guidelines, Example 10 at page 39 (emphasis added).

Given the level of skill in the art, specifying highly stringent conditions leads to "no substantial variation within the [claimed] genus," and therefore a skilled artisan would recognize that the Applicants were in possession of the necessary common attributes or features of the genus. The common element or attribute of the claimed genus is that species of the genus are structurally related to SEQ ID NO:8, such that they hybridize to SEQ ID NO:8 or the related sequences under the specified high stringency conditions recited in the claims.

Applicants submit that the skill in the art is such that the sequence of nucleic acids which hybridize to SEQ ID NO:8 under the conditions specified can be conceived. Here, the claimed genus is defined by its structure – members of the genus hybridize under the specified conditions to the specified sequences, each of which are adequately described in the specification.

Applicants submit that the pending claims relating to nucleic acids encoding polypeptides having 80% to 99% sequence identity to the amino acid sequence of the polypeptide of SEQ ID NO: 9 with the functional recitation "wherein said isolated nucleic acid encodes a polypeptide having the ability to induce c-fos expression" are also adequately described. In Example 14 of the written description training materials, the written description requirement was found to be satisfied for claims relating to polypeptides having 95% homology to a particular sequence and possessing a particular catalytic activity, even though the applicant had not made any variants. Similarly, the pending claims also have very high sequence homology to the disclosed sequences and must share the same activity *in vivo*. In Example 14, the procedures for making variants were known in the art and the disclosure taught how to test for the claimed catalytic activity. Similarly, in the instant application, it is well known in the art how to make nucleic acids encoding polypeptides which have at least 80% sequence identity to the disclosed sequences, and

the specification discloses how to test to determine if the encoded polypeptides induce *c-fos* expression. Like Example 14, the genus of nucleic acids encoding polypeptides that have at least 80% or 99% sequence identity to the disclosed amino acid sequences will not have substantial variation since all of the variants must have the same ability to induce *c-fos*.

Furthermore, while Applicants appreciate that actions taken by the PTO in other applications are not binding with respect to the examination of the present application, Applicants note that the PTO has issued many patents containing claims to variant nucleic acids or variant proteins where the applicants did not actually make such nucleic acids or proteins. Representative patents include U.S. Patent No. 6,737,522, U.S. Patent No. 6,395,306, U.S. Patent No. 6,025,156, U.S. Patent No. 6,645,499, U.S. Patent No. 6,498,235, and U.S. Patent No. 6,730,502, which are attached hereto as Exhibits 2-7).

In conclusion, Applicants submit that they have satisfied the written description requirement for the pending claims based on the actual reduction to practice of SEQ ID NO:8 and SEQ ID NO: 9, by specifying the high stringency conditions under which hybridization occurs, and by describing the *c-fos* induction assay, all of which result in a lack of substantial variability in the species falling within the scope of the instant claims. Applicants submit that this disclosure would allow one of skill in the art to "recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus." Hence, Applicants respectfully request that the PTO reconsider and withdraw the written description rejection under 35 U.S.C. §112.

## CONCLUSION

In view of the above, Applicants respectfully maintain that claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: June 15, 2005

By: _____
AnneMarie Kaiser
Registration No. 37,649
Attorney of Record
Customer No. 30,313
(619) 235-8550

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EXHIBIT 1

APMIS 112: 463–80, 2004
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APMIS
ISSN 0903-4641

# Molecular regulation of the VEGF family – inducers of angiogenesis and lymphangiogenesis

BRADLEY K. McCOLL, STEVEN A. STACKER and MARC G. ACHEN

Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Victoria, Australia

McColl BK, Stacker SA, Achen MG. Molecular regulation of the VEGF family – inducers of angiogenesis and lymphangiogenesis. APMIS 2004;112:463–80.

The vascular endothelial growth factor (VEGF) family of secreted glycoproteins are critical inducers of angiogenesis (growth of blood vessels) and lymphangiogenesis (growth of lymphatic vessels). These proteins are attractive therapeutic targets for blocking growth of blood vessels and lymphatics in tumors and thereby inhibiting the growth and spread of cancer – in fact, the first VEGF inhibitor has recently entered the clinic for treatment of cancer. In addition, the VEGFs are being considered for stimulation of angiogenesis in the context of ischemic disease and lymphangiogenesis for treatment of lymphedema. These therapeutic possibilities have focused great interest on the molecular regulation of VEGF family members. Much has been learned in the past five years about the mechanisms controlling the action of the VEGFs, including the importance of hypoxia, proteolysis, transcription factors and RNA splicing. An understanding of these mechanisms offers broader opportunities to manipulate expression and activity of the VEGFs for treatment of disease.

Key words: VEGF-C; VEGF-D; lymphatics; proteolysis.

Marc G. Achen, Ludwig Institute for Cancer Research, PO Box 2008, Royal Melbourne Hospital, Victoria 3050, Australia. e-mail: Marc.achen@ludwig.edu.au

Angiogenesis and lymphangiogenesis – the development of new blood vessels and lymphatics from the pre-existing vasculature, respectively – are processes with integral roles in embryonic development and numerous diseases. Angiogenesis is the better understood of the two processes, in part due to the intense research focus placed upon the field because of the significance of blood vascular development for tumor growth and ischemic disease. Angiogenesis research has progressed to the point where the first molecular therapies targeting nascent blood vessels in cancer are reaching the clinic (1–4).

A lack of molecular markers specific to the lymphatic system has been an impediment to lymphangiogenesis research until recently, when the identification of several such markers (reviewed in (5, 6)) has led to molecular insights into lymphangiogenesis. Numerous pathologies are associated with the lymphatics, such as the metastatic spread of cancer and lymphedema, and therapeutic strategies based upon the expanding body of lymphatic knowledge are now being considered (7–9).

The development of blood vessels and lymphatics depends upon interactions between the vascular endothelium and signalling molecules derived from the serum and extracellular matrix (ECM). Among these signalling molecules, the vascular endothelial growth factor (VEGFs) family of proteins is central to angiogenesis and lymphangiogenesis. The VEGFs are dimeric endothelial cell mitogens encoded by five genes in mammals: *VEGF* (also known as *VEGF-A*), *VEGF-B*, *VEGF-C*, *VEGF-D* and placenta growth factor (*PlGF*) (10). Members of the fam-

Invited Review.

ily are related by the characteristic VEGF homology domain (VHD), containing receptor binding sites and a conserved cystine-knot motif (10, 11). Variations in mRNA splicing generate isoforms of several of the VEGFs, adding to the complexity of the family (12–21). Recent work has advanced the understanding of the functions of the VEGFs, and uncovered some of the mechanisms by which they are regulated. This review will discuss the molecular and physiological stimuli controlling the expression and activity of the VEGF family in healthy and diseased tissues.

## THE BLOOD VASCULATURE

### *VEGFs in embryonic development and physiological angiogenesis*

*VEGF.* Embryonic development of the blood vascular system commences with the process of vasculogenesis, whereby endothelial cell precursors differentiate to endothelial cells and associate to form the primary vascular plexus (22, 23). Subsequent angiogenesis gives rise to a more extensive vasculature (22, 23). In the healthy adult, angiogenesis is chiefly restricted to wound healing and the female reproductive system. Postnatal neo-vascularization has been shown to make use of endothelial precursor cells from the bone marrow, which are incorporated into the nascent vessels (24, 25). New vessels are therefore not exclusively derived from the cells of pre-existing vessels.

The essential role of VEGF for embryonic vasculogenesis has been established via gene disruption studies. Disruption of a single *VEGF* allele causes embryonic lethality by embryonic day (E) 11 as a result of defective vasculogenesis, angiogenesis and large vessel formation (26, 27). Postnatal ablation of *VEGF* causes death in mice up to approximately 4 weeks of age due to defects including impaired organ development and reduced vascularisation, probably stemming from reduced angiogenesis (28). Disruption of VEGF signalling is apparently not harmful after this stage, although corpus luteum angiogenesis in the adult is VEGF dependent (29). VEGF therefore appears to have a limited role in maintaining the mature vasculature, instead impacting on those systems with an ongoing dependence upon angiogenesis.

Examination of the postnatal vascularisation of the retina reveals the mechanism whereby new vessels are stabilised and made VEGF-independent. Newly formed vessels in the retina undergo regression in the absence of VEGF (30), as a result of endothelial cell apoptosis. Temporal studies of the developing retinal vasculature reveal that formation of the vascular plexus precedes recruitment of pericytes to the new vessels, and pericyte association is crucial for stabilisation of the neo-vasculature (31). Therefore VEGF is necessary to maintain immature vessels and, without full pericyte coverage, new vessels will regress in the absence of VEGF stimulation.

The effects of VEGF are mediated by binding to VEGFR-1 and VEGFR-2, receptor tyrosine kinases expressed on the blood vascular endothelium (Fig. 1). Both VEGFR-1 and VEGFR-2 are essential for embryonic vascular development and viability, although differences in the phenotypes of mice lacking the receptors reveal distinct functions. VEGFR-2 is believed to be the principal pro-angiogenic receptor, as deletion of the *VEGFR-2* gene results in failed vasculogenesis with a lack of organised vessels and haematopoietic precursors (32). VEGFR-1 ablation causes defective assembly of endothelial cells (33), related to the development of increased numbers of hemangioblasts (34). The VEGFR-1 intracellular tyrosine kinase domain is not essential for embryonic viability (35), sug-

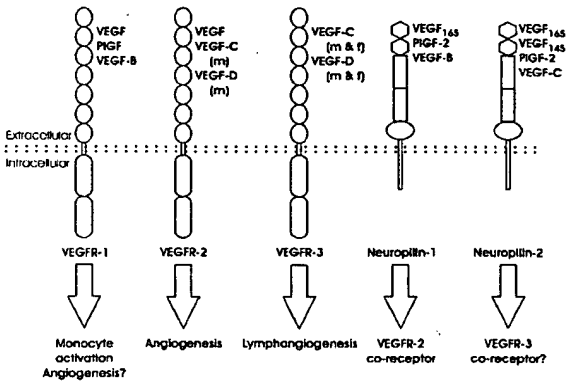


*Fig. 1.* Interaction of the VEGFs with receptors. Ligands for each receptor are listed. Where isoforms are not specified, all isoforms bind the receptor. Mature and full-length forms of VEGF-C and VEGF-D are denoted "m" and "f", respectively. The proposed function is listed below each receptor. The role of VEGFR-1 in angiogenesis is uncertain.

gesting that VEGFR-1 may be a negative regulator of VEGF activity.

Several isoforms of VEGF exist, generated by alternative mRNA splicing (12–16). The smallest isoform, $VEGF_{121}$ is freely soluble, whereas $VEGF_{189}$ and $VEGF_{206}$ are completely bound to the ECM (36). The predominant isoform, $VEGF_{165}$, exists as both soluble and ECM-bound forms. Emerging data show specific functions for the VEGF isoforms (37), possibly relating to the isoform-specific binding of VEGF to a second class of receptors, the Neuropilins. Neuropilin-1 (NRP-1) and Neuropilin-2 (NRP-2) are cell surface receptors associated with neuronal guidance (38). $VEGF_{165}$ binds both NRP-1 and NRP-2, but $VEGF_{145}$ binds NRP-2 only (39–41). Overexpression of the *NRP-1* gene is lethal at the embryonic stage, resulting in excessive capillary and blood vessel formation, thinning of the walls of the heart, and defects in other systems (42). Likewise, deletion of *NRP-1* causes death due to defects in embryonic vascularisation, although at later stages than those seen in VEGFR-2 deficient mice (43). *NRP-2* is not required for viability, but disruption of both *NRP-1* and *NRP-2* genes results in embryonic death due to severe vascular abnormalities, similar to those of VEGF or VEGFR-2 deficient mice (44). Furthermore, co-expression of NRP-1 with VEGFR-2 suggests that NRP-1 enhances VEGF binding to VEGFR-2 (40). NRP-1 may therefore function in concert with VEGFR-2 as a VEGF co-receptor during angiogenesis.

Despite the function of VEGF as a primary inducer of angiogenesis, some studies of VEGF-mediated initiation of blood vessel growth suggest that this growth factor alone is insufficient to establish mature vessels. Studies in which VEGF is elevated, in muscle from engineered myoblasts (45), transgenically in the skin (46) or systemically using adenoviral vectors (47) resulted in vascular leakage with associated inflammation. It seems that additional signalling molecules, most notably the Angiopoietins, their receptors the Ties, and the Ephrins are also necessary to give rise to fully functional vessels (23, 48–50).

*VEGF-B.* Although closely related to VEGF (19, 51), the physiological function of VEGF-B is less clear. Alternative RNA splicing generates two isoforms of VEGF-B, $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$ (20), the larger of which is the predominant mRNA species (52). Both isoforms are ligands for VEGFR-1 and NRP-1 (Fig. 1), although $VEGF\text{-}B_{186}$ requires proteolytic cleavage of the C-terminal region for binding to NRP-1 (53) (Fig. 2). Neither isoform binds VEGFR-2 or VEGFR-3 (53, 54). VEGF-B induces a mitogenic response in endothelial cells *in vitro* (51), and expression of VEGF-B in the embryonic heart and adult cardiac and skeletal muscle suggests a role in vascularisation of the musculature (51). However, VEGF-B does not appear to be strongly angiogenic as indicated by adenoviral delivery to periadventitial tissue (55) or hindlimb skeletal muscle (56). Unlike VEGF, mice lacking VEGF-B are viable, but suffer from smaller hearts, abnormal coronary vasculature and defective recovery from cardiac ischemia (57). Certain VEGF isoforms are also required for development of the heart (37), and coexpression studies have shown that VEGF and VEGF-B can form heterodimers (51). Together these data indicate a role for VEGF-B in cardiac development, possibly in cooperation with VEGF.

*PlGF.* PlGF is expressed in the placenta throughout pregnancy, and also in the heart, lung, brain and skeletal muscle (58, 59). Alternative splicing of the human primary transcript generates three isoforms: $PlGF_{131}$ (PlGF-1), $PlGF_{152}$ (PlGF-2) and $PlGF_{203}$ (PlGF-3) (17, 18). Like VEGF-B, PlGF is a ligand for VEGFR-1 but not VEGFR-2 (58, 60), and PlGF-2 is able to bind NRP-1 and NRP-2 (61, 62) (Fig. 1). PlGF homodimers are poor inducers of angiogenesis *in vivo*, and only provoke a weak mitogenic response from endothelial cells (60, 63). VEGF can form heterodimers

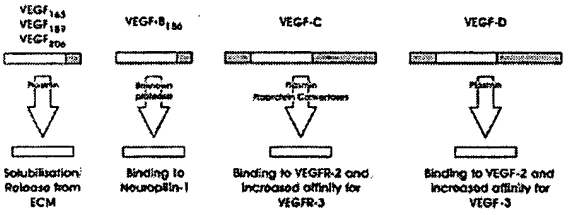


*Fig. 2.* Proteolytic activation of the VEGFs. Shading represents regions of the protein removed by proteolysis. Functional outcomes of proteolysis are listed below each growth factor.

with PlGF(63), although conflicting evidence exists as to the capacity of such heterodimers to induce angiogenesis (63, 64). Deletion of the *PlGF* gene in mice does not cause significant defects in vascular development (65), although overexpression of *PlGF* in the skin results in increased vessel formation and permeability (66), demonstrating a pro-angiogenic role for the growth factor. PlGF stimulates angiogenesis as well as migration and proliferation of endothelial cells *in vitro* in a manner dependent upon VEGF and VEGFR-1 (65). A model has been suggested to explain these observations (65), in which PlGF binding to VEGFR-1 signals for angiogenesis, whereas VEGF binding does not. PlGF binding to VEGFR-1 would displace VEGF from the receptor, increasing the amount of soluble VEGF available for pro-angiogenic binding to VEGFR-2. The VEGFR-1 tyrosine kinase domain is not essential for embryonic vascular development (35), indicating that VEGFR-1 acts as a non-signalling receptor which is consistent with the normal vascular development observed in PlGF deficient embryos (65).

*In vivo* studies of ischemia have shown that PlGF treatment stimulates arteriogenesis, the enlargement of pre-existing arterioles (67, 68). Ablation of the *PlGF* gene inhibits this process, a phenotype which can be rescued by bone marrow transplantation (69). PlGF is capable of stimulating migration and tissue factor production in monocytes (70), and PlGF treatment results in elevated extravasation of macrophages (68). These lines of evidence concur with the observed contribution of bone marrow derived endothelial cell precursors to vessel formation (25), indicating a role for haematopoietic cells in angiogenesis, in response to stimulation by PlGF.

*Pathological angiogenesis*

Aberrant or insufficient vascularisation of tissues is a feature of several pathologies, including arthritis, atherosclerosis, diabetic retinopathy and tumor growth. In the case of cancer, access to the blood supply and the nutrients therein is a necessary precursor for significant tumor growth, making tumor angiogenesis a target for emerging therapeutics (71–74). In contrast to developmental angiogenesis, tumor angiogenesis results in irregularly shaped, convoluted vessels that exhibit disorganised or even reversed blood flow (73). Furthermore, tumor derived vasculature is often leaky, a characteristic of immature blood vessels (75). Tumor angiogenesis may incorporate cancer cells into the wall of the developing vessel (76), and tumor models have been reported in which vessel growth involves the recruitment of circulating endothelial precursor cells, suggesting that new vessels are not solely derived from pre-existing vasculature (25). It has also been hypothesised that some tumor cell types may form vessel-like structures independently of the endothelium (77). Tumor angiogenesis therefore appears to be a somewhat chaotic process, in which normal signalling molecules are co-opted in a disorganised manner to provide the tumor with improved access to the blood supply (73).

The evidence for VEGF as a primary inducer of tumor angiogenesis is extensive. Animal xenograft models of cancer in which the *VEGF* gene has been disrupted, or VEGF signalling blocked, show drastically reduced growth of tumors, demonstrating the significance of VEGF in tumor growth (27, 78–80). Surveys of human cancers reveal significant elevation of VEGF expression in the majority of tumors examined, including those from the lung, breast, gastrointestinal tract, ovary and colon (81–85). Expression of VEGF correlates with increased tumor vascularisation and poor patient prognosis (81, 86, 87), suggesting VEGF as a therapeutic target, and leading to the development of therapies based upon inhibition of VEGF activity. The approval of the US Food and Drug Administration of Avastin™, a neutralizing antineutralizing antibody to VEGF, for treatment of metastatic colorectal cancer has focussed great interest on the field of anti-angiogenesis as an approach to cancer therapy (1, 4).

PlGF may be associated with pathological angiogenesis as demonstrated by decreased vascularisation of tumor models lacking PlGF (65), although expression of PlGF has been reported to be variable in human cancers (88–90). Inhibition of the PlGF receptor VEGFR-1 suggests a role for PlGF in tumor angiogenesis as well as arthritis and atherosclerosis (67), possibly through synergistic effects with VEGF (reviewed in (91)). Examination of VEGF-B in tumors reveals little conclusive data to indicate a substantial role for VEGF-B in tumor progression (92, 93), although isoform specific vari-

ation of VEGF-B expression in tumors has been reported (52).

*Molecular regulation of angiogenic VEGFs*

*VEGF*

*Hypoxia.* Cellular growth and proliferation beyond the dimensions through which oxygen can readily diffuse results in reduced oxygen tension in the tissue. Within the cell, a number of metabolic alterations occur in response to hypoxia, including the synthesis of pro-angiogenic growth factors targeting the vascular endothelium, most notably VEGF (94). Hypoxia has been demonstrated to be a major regulator of VEGF both *in vitro* and *in vivo* (95–97) (Table 1), and VEGF mRNA is elevated in ischemic tumor cells adjacent to areas of necrosis (98). These observations have led to efforts to identify inhibitors of the hypoxic response as novel cancer therapeutics (99).

The cellular response to hypoxia is mediated by the hypoxia-inducible transcription factor (HIF-1), a heterodimeric protein which binds to hypoxia response elements (HRE) in the promoter/regulatory regions of hypoxia-inducible genes, including the *VEGF* gene, and initiates transcription by recruitment of transcriptional activators such as CREB/p300 (100). Under normoxic conditions, the α subunit of HIF-1 is degraded via the ubiquitin-proteasome pathway, preventing formation of active HIF-1 (for review of HIF-1 regulation see (101)). The ubiquitination step is mediated by the von Hippel-Lindau (VHL) protein (102), which recognises hydroxylated proline residues in HIF-1α (101). Proline hydroxylation of HIF-1α is catalysed by a family of three prolyl hydroxylases utilising molecular oxygen as a substrate (103). Studies of recombinant prolyl hydroxylases demonstrate that the affinity of these enzymes for oxygen is such that their activity is reduced at oxygen concentrations observed in hypoxic tissues (104), leading to reduced ubiquitination-degradation of HIF-1α. Hydroxylation of an asparagine residue also regulates the activity of the C-terminal transactivation domain of HIF-1α by blocking interactions with the transcriptional activator p300 (105). As with the prolyl hydroxylases, activity of the enzyme responsible is dependent upon oxygen concentration at physiological levels (106, 107). Hydroxylation of HIF-1α therefore acts as a hypoxia sensitive switch linking local oxygen concentrations to the stability and activity of the transcription factor, and hence to VEGF expression.

*Genes associated with transformation.* Numerous genes associated with tumorigenic transformation are also involved with regulation of VEGF expression (Table 1). Examination of the *VEGF* gene promoter region reveals the presence of consensus AP-1 sequences, sites recognised by the c-fos family of transcription factors (15). The c-fos family are proto-oncogenes which activate transcription by binding to AP-1 sites in transcriptional promoters (for review see (108)). Since fos is upregulated by treatment with phorbol esters and various growth factors, VEGF expression could also be elevated in response to these stimuli, as is indeed the case (15, 109–112).

The *ras* oncogene is another logical candidate to activate VEGF expression, as ras activity is a

TABLE 1. *Regulators of the VEGFs. Arrows indicate positive (↑) or negative (↓) stimuli*

| VEGF | PIGF | VEGF-B | VEGF-C | VEGF-D |
|---|---|---|---|---|
| Regulation of transcription | | | | |
| ↑ Hypoxia | | | | |
| ↑ Inflammatory mediators/ COX-2 | ↑ Hypoxia | | ↑ Inflammatory mediators/ COX-2 | ↑ AP-1 transcription factors |
| ↑ AP-1 transcription factors | ↑ FoxD1 transcription factor | | | ↑ Cell-cell contact |
| ↑ Mutant ras | | | | |
| ↑ Mutant p53 | | | | |
| Post-transcriptional regulation | | | | |
| ↑ Proteolysis (release from ECM) | | ↑ Proteolysis | ↑ Proteolysis | ↑ Proteolysis |
| | | | | ↓ Cytosolic β-catenin |

component of the signal transduction pathway linking extracellular stimuli to AP-1 mediated activation of transcription (108). Oncogenic, activated ras stimulates VEGF expression in cell lines (113, 114), and ras activity is associated with VEGF expression, tumorigenicity and angiogenesis in mouse models of cancer (115, 116). Therefore oncogenic ras may play a role in stimulating angiogenesis as well as driving proliferation of tumor cells in cancer.

A transcriptionally inactive mutant of the tumor suppressor p53 causes elevated VEGF expression (117), and wild-type p53 represses VEGF transcription (118). Deletion of the *p53* gene in tumor cells results in enhanced vascularisation and tumor growth, and p53-deficient cells show increased induction of VEGF under hypoxic conditions (119). The p53 protein promotes ubiquitination and degradation of HIF-1α (119), hence VEGF expression is enhanced by HIF-1 in the absence of p53. In addition to upregulating VEGF, mutation of p53 may confer resistance to antiangiogenic therapies, as hypoxia-induced apoptosis is p53 dependent. A p53 mutant tumor model has shown reduced responsiveness to therapies targeting the vasculature (120), demonstrating that p53 status may be a factor when considering anti-VEGF therapies.

*RNA splicing isoforms and proteolysis.* VEGF exists as a number of isoforms derived from mRNA splice variants (12, 14–16). The isoforms differ at the C terminus, with the larger variants exhibiting substantial affinity for heparin (36). The smallest isoform, $VEGF_{121}$, does not bind heparin and is freely soluble upon secretion, whereas the higher molecular weight isoforms bind to the cell surface/extracellular matrix (ECM) (36, 121). VEGF isoforms sequestered in this way can be released from the ECM by the action of heparinase or the fibrinolytic serine protease plasmin, giving rise to soluble, biologically active molecules (36, 121) (Fig. 2). It has been proposed that the ECM-bound forms of VEGF represent a pool of available growth factor, which can be activated in the course of tissue remodelling in conjunction with the degradation of ECM components necessary for neovascularisation (121).

*Inflammatory cytokines and cyclooxygenase-2 (COX-2).* The inflammatory condition rheumatoid arthritis is characterised by proliferation of the synovium, including large numbers of blood vessels, and invasion of the adjacent cartilage. Angiogenesis is a significant component of this process, believed to be driven by local production of angiogenic growth factors (91). Inflammatory mediators such as IL-1α, IL-1β, TGF-β and prostaglandin E2 (PGE2) induce VEGF expression in a number of cell types, including umbilical vein endothelial cells, smooth muscle cells and synovial fibroblasts (111, 122–125). VEGF is therefore a potential target for intervention in inflammatory disease. Furthermore, the enzyme COX-2 is an inducible component of the inflammatory prostaglandin synthesis pathway, and is upregulated in many human cancers (126). Inhibitors of the enzyme demonstrate anti-angiogenic activity in animal models of cancer, suggesting that COX-2 activity may be a target for therapies acting indirectly on VEGF (127, 128).

*PlGF and VEGF-B*

Like VEGF, PlGF is also induced by hypoxia (129), and recent work has shown *PlGF* expression to be activated by FoxD1, a member of the Forkhead/Winged Helix family of transcription factors associated with branching of the ureteric bud in the kidney (130). *PlGF*-null mice have not been reported to develop renal defects, indicating either a non-essential or redundant role for PlGF in the kidney. In addition, PlGF is upregulated in keratinocytes during wound healing (65, 131). The three human isoforms of PlGF differ in their affinities for the ECM, with PlGF-2 incorporating a sequence of 21 amino acids necessary for binding to heparin (17, 132). VEGF and PlGF are therefore both regulated according to tissue oxygenation and matrix interactions, demonstrating the interplay between vascular development and the ECM.

VEGF-B does not respond to hypoxia, Ras oncoprotein or several other stimuli known to regulate other VEGF family members (112, 133, 134), and the mechanisms whereby *VEGF-B* gene expression is regulated remain unclear.

## THE LYMPHATIC VASCULATURE

The lymphatic vasculature is composed of a network of blind-ended, thin-walled vessels and

capillaries lined by a continuous layer of endothelial cells (135). Extravasated fluid and solutes from the extracellular spaces are collected by the initial lymphatics, then passed through a network of progressively larger lymphatic vessels, finally returning to the blood circulation via the thoracic duct (136). In addition, the lymphatic system is a major site of immune surveillance as stimulated dendritic cells migrate to the lymph nodes where foreign antigens are presented to lymphocytes (137).

*Embryonic development and physiological lymphangiogenesis*

The lymphatic vessels develop subsequent to the formation of the blood vasculature, leading to the suggestion that they are derived from the blood vessels (138). Recent advances in the understanding of lymphatic development have shown that expression of the homeobox transcription factor Prox-1 by a population of endothelial cells in the cardinal vein during embryogenesis is a defining characteristic of differentiation to the lymphatic phenotype (139, 140). Subsequent budding and sprouting of the Prox-1 positive cells gives rise to the lymphatic vasculature (for review see (141)).

Two members of the VEGF family, VEGF-C and VEGF-D, have been shown to act as lymphangiogenic growth factors (142, 143). Expression of either growth factor in the skin of transgenic mice results in lymphatic hyperplasia without altering the blood vasculature (143, 144). Disruption of the *VEGF-C* gene demonstrates that the growth factor is indispensable for embryonic lymphangiogenesis (145). Embryos carrying a homozygous deletion of *VEGF-C* are not viable, and fail to form the initial lymph sacs which generate the lymphatic vasculature. Prox-1 expression was detected in endothelial cells of VEGF-C deficient embryos, but the Prox-1 positive cells failed to migrate from the cardinal vein (145), indicating that VEGF-C is required for migration of the endothelial cells which go on to form the lymphatic system. *VEGF-C* exhibits a gene dosage effect as mice carrying only one functional *VEGF-C* allele are prone to lymphedema (145). The role of VEGF-D during embryonic development is unknown.

In the human, VEGF-C and VEGF-D are ligands for VEGFR-2 and the related receptor tyrosine kinase VEGFR-3 (146, 147), although mouse VEGF-D binds only VEGFR-3 (148). In the early embryo VEGFR-3 is broadly expressed on the endothelium, but in time becomes restricted to the lymphatic endothelium (149), leading to the suggestion that VEGFR-3 signals for lymphangiogenesis. Functional studies support this hypothesis, demonstrating that VEGFR-3 transduces mitogenic and migratory signals in lymphatic endothelial cells, and stimulation of the receptor blocks apoptosis induced by serum starvation (150). Furthermore, expression in the skin of a mutant form of VEGF-C specific for VEGFR-3 causes lymphatic hyperplasia without altering blood vessel structure (143), and disruption of VEGFR-3 signalling in the same model blocks development of the lymphatic phenotype (143). VEGFR-3 is therefore a major transducer of lymphangiogenic signalling in the adult. In addition, deletion of the *VEGFR-3* gene results in embryonic death due to defective remodelling of the large blood vessels (151). VEGFR-3 therefore has a role in embryonic vascular development prior to the emergence of the lymphatics.

VEGF-C has also been shown to bind to NRP-2 (152), and deletion of the *NRP-2* gene impairs formation of the small lymphatics (153), leading to the hypothesis that NRP-2 may act as a co-receptor for VEGFR-3, in a fashion analogous to NRP-1 acting as a co-receptor for VEGFR-2 (Fig. 1).

Examination of VEGF-C and VEGF-D function in a number of assays has also shown an angiogenic activity for the growth factors (154–158), presumably via activation of VEGFR-2. Gene delivery experiments using adenoviruses expressing VEGFs show induction of angiogenesis by VEGF-C and VEGF-D *in vivo* (55, 56). Although the physiological significance of VEGF-C and VEGF-D in angiogenesis is unclear the promising results achieved so far with gene delivery approaches suggest a role for the factors in therapy for ischemic disease.

*Pathological lymphangiogenesis and lymphatic dysfunction*

*Cancer.* Given the propensity for numerous types of cancer to metastasize via the lymphatic system, it has been proposed that tumors may stimulate lymphangiogenesis in a manner analogous to tumor angiogenesis, thereby promot-

ing lymphogenous metastasis (6, 159). The production of lymphangiogenic growth factors is believed to stimulate lymphatic vessel development in the region of the tumor, enabling cancer cells to gain access to the lymphatic vasculature.

A number of animal models have been utilised to examine the potential of VEGF-C and VEGF-D to promote lymphatic metastasis. Studies in which VEGF-C or VEGF-D were expressed in transplanted tumor cells or transgenic tumor models have demonstrated that these growth factors promote tumor lymphangiogenesis and lymphatic metastasis (160–164). Furthermore, clinicopathological studies of these lymphangiogenic growth factors in cancer reveal that, in many instances, expression of VEGF-C or VEGF-D does indeed correlate with the capacity of a tumor to metastasize (165–170). Analysis of xenograft tumor models has revealed that VEGF-C and VEGF-D can also drive tumor angiogenesis and accelerate solid tumor growth (158, 164), potentially as a result of VEGFR-2 activation. For a complete discussion of VEGF-C and VEGF-D and tumor lymphangiogenesis, see Stacker et al., this issue.

*Lymphedema.* A spectrum of pathologies exists stemming from inadequate lymphatic function (for review of disorders of the lymphatic system see (135, 171)). Although sharing the common feature of fluid accumulation in tissues as a result of inadequate lymphatic drainage (lymphedema), the underlying causes may be either familial or acquired. Specific genetic lesions have been identified in a few instances of inherited lymphedema, providing insights into lymphangiogenesis. Specifically, mutations in the *VEGFR-3* gene have been identified in a number of cases (172, 173), and the forkhead transcription factor FOXC2 is also implicated in congenital lymphedema (174). Acquired lymphedema can be the result of infection (commonly filariasis) or lymph node resection and radiation treatment of cancer. Regardless of the cause, current treatments for lymphedema are targeted towards alleviating symptoms rather than treating the underlying causes (171). A better understanding of the molecular control of lymphangiogenesis offers the potential to develop novel treatments for this painful and disfiguring condition based on stimulating lymphatic development to enhance or restore lymphatic function (7, 8).

*Molecular regulation of lymphangiogenic VEGFs*

*Proteolytic activation of VEGF-C and VEGF-D.* VEGF-C and VEGF-D are both synthesised as proproteins, with the central receptor binding VEGF homology domain (VHD) flanked by N- and C-terminal propeptides (146, 147). During biosynthesis the propeptides are cleaved off, yielding the mature VHD (175, 176). As a consequence of proteolysis VEGF-C acquires the capacity to bind to VEGFR-2 (175) and a recombinant mature form of VEGF-D shows an approximately 290-fold greater affinity for VEGFR-2 than unprocessed VEGF-D (176). Full-length forms of both growth factors bind to VEGFR-3, but do so with greater affinity after proteolytic maturation (175, 176). In support of these observations, mutation of one of the sites of proteolytic cleavage in VEGF-C has been shown to reduce lymphangiogenesis and angiogenesis induced by VEGF-C in a mouse tumor model (177).

The capacity of the mature forms of VEGF-C and VEGF-D to act as ligands for VEGFR-2 explains how these proteins stimulate angiogenesis in addition to lymphangiogenesis (156–158, 164). The proteolysis-dependent binding to VEGFR-2 may represent a mechanism whereby the angiogenic activity of VEGF-C and VEGF-D is activated.

The marked effect of the proteolytic activation of VEGF-C and VEGF-D on their affinity for VEGFR-2 and VEGFR-3 indicates that the enzymes carrying out this processing are important regulators of lymphangiogenesis and angiogenesis. Treatment of VEGF-C and VEGF-D with the fibrinolytic serine protease plasmin was recently shown to generate fully processed, mature forms of the VHD with greatly enhanced capacities to activate both VEGFR-2 and VEGFR-3 (178). Since plasmin can also release ECM-bound VEGF, this enzyme is capable of activating both angiogenic (36) and lymphangiogenic growth factors (Fig. 2). These findings suggest that plasmin is a master regulatory molecule that co-ordinates lymphangiogenesis, angiogenesis and fibrinolysis during wound healing.

Some members of the furin/proprotein con-

vertase (PC) family of proteases have also been shown to partially activate VEGF-C (177). The PCs are a family of ubiquitously expressed proteases capable of activating a range of precursor proteins by cleavage downstream of dibasic residues. Substrates for the PCs include numerous growth factors, zymogens, receptors and viral proteins (for review of PC function see (179, 180)). The PCs furin, PC5 and PC7 are able to cleave VEGF-C at the C-terminal site of proteolysis, thereby cleaving the C-terminal propeptide from the VHD (177). However, it is not known if PCs cleave the N-terminal propeptide from the VHD, nor if they process VEGF-D.

These observations suggest a model in which VEGF-C and VEGF-D may be activated in various biological contexts. Firstly, regulated expression of PCs could activate VEGF-C and VEGF-D during embryogenesis, when plasmin is unlikely to play a role (note that mice deficient for plasminogen, the plasmin precursor, are viable and have not been reported to suffer lymphedema (181)). Plasmin could activate VEGF-C and VEGF-D from a pool of inactive full-length molecules in response to tissue damage in adults. It is known that both VEGF-C and VEGF-D are localised in vascular smooth muscle in adult human tissues (182, 183). Local activation of these molecules in response to tissue damage and plasmin production could represent a mechanism for stimulating rapid vessel repair during wound healing. In addition, activation of VEGF-C and VEGF-D by plasmin may be associated with tumor lymphangiogenesis, given that plasminogen deficient mice show delayed formation of lymph node metastases when inoculated with Lewis lung carcinoma cells (184). Upregulation of VEGFR-2 and VEGFR-3 on blood vessels during wound healing and tumor angiogenesis (185) may facilitate the development of new vessels.

*Induction of VEGF-C expression by inflammatory cytokines.* Interleukin-1 (IL-1) and Tumor Necrosis Factor-α (TNF- α) stimulate VEGF-C expression in human lung fibroblasts and human umbilical vein endothelial cells (HUVEC) (186). In conjunction with inflammation-induced VEGF expression and angiogenesis, expression of VEGF-C and consequent lymphangiogenesis may have a role in maintaining fluid balance in the inflamed tissue. Also, an immune response at the site of inflammation may depend upon increased lymphatic function in the affected area. It therefore seems logical that inflammatory mediators can induce expression of a lymphangiogenic growth factor such as VEGF-C.

*VEGF-C and COX-2.* The association of VEGF-C with the inflammatory response extends to COX-2. Recent work has shown COX-2 to be capable of upregulating VEGF-C expression in human lung adenocarcinoma cells (187). In addition to VEGF-C, COX-2 activity and some prostaglandins produced by COX-2 (188) also elevate angiopoietin-2, another protein required for lymphangiogenesis (189). COX-2 is therefore an inducer of two proteins integral to lymphangiogenesis. This relationship has significant implications for tumor lymphangiogenesis and metastasis, given the earlier observation that elevated COX-2 expression may be associated with lymph node metastasis from adenocarcinoma of the lung (190). COX-2 may therefore be a major inducer of angiogenic and lymphangiogenic proteins, and a potential target for anti-metastatic therapies based upon existing anti-inflammatory compounds.

*VEGF-D induction by fos.* The mouse homologue of *VEGF-D* was originally identified as a novel c-fos responsive gene (191), and subsequent investigation of the X-linked human *VEGF-D* gene (192) revealed a canonical AP-1 binding site in the transcriptional promoter (192, 193). It has been reported that VEGF-D levels are high in glioblastoma multiforme (GBM), yet levels of c-fos are very low in this tumor (194). However, the fos-related antigen-1 (fra-1), another AP-1 transcription factor, is elevated in GBM and induces VEGF-D expression (194). Therefore, the AP-1 transcriptional activation pathway appears to be a regulator of VEGF-D expression. Although the significance of this relationship in healthy physiology is unclear, the association between increased transcription mediated by AP-1 and tumorigenesis is well established (108). Therefore, elevated AP-1 activity may lead not only to neoplasia, but also to tumor angiogenesis and lymphangiogenesis via elevated VEGF-D expression.

*VEGF-D and cell-cell contact.* The cadherins are a family of calcium-dependent cell surface pro-

teins which mediate cell-cell adhesion through homotypic binding with their counterparts on adjacent cells (195). VEGF-D expression has been shown to be enhanced by cadherin-11 *in vitro* whereas inhibition of cadherin-11 expression reduces VEGF-D induction (196). In addition, VEGF-D mRNA stability is downregulated by cytosolic β-catenin (197). β-catenin exists either as a structural protein at the cell membrane in a complex with the cadherins, or in the cytoplasm. Repression by cytosolic β-catenin and upregulation in response to cadherin-11 implies that VEGF-D expression is dependent upon stable cellular interactions with the surrounding environment, and may have implications for the secretion of lymphangiogenic growth factors by migrating cells.

## CONCLUDING REMARKS

Numerous members of the VEGF family share common mechanisms of regulation. Regulating the angiogenic and lymphangiogenic activities of the VEGFs via common pathways may allow coordinated development of the lymphatic and blood vasculature, necessary for fluid homeostasis.

The existence of multiple protein isoforms generated by variations in RNA splicing has been reported for all mammalian VEGFs except VEGF-C. In the case of VEGF (14–16), VEGF-B (19, 20) and PlGF (17, 18) the generation of isoforms with different affinities for heparin and the ECM/cell surface regulates the biological distribution of these molecules. However, the significance of the two isoforms of mouse VEGF-D (21), that differ in the C-terminal propeptide, is as yet unknown.

Proteolysis regulates several VEGFs (Fig. 2), and significantly influences receptor affinity of one isoform of VEGF-B (53), and receptor affinity and specificity of VEGF-C (175) and VEGF-D (176). Proteolysis also modulates VEGF activity, by releasing ECM-bound forms of VEGF (36). The findings that plasmin can activate both VEGF-C and VEGF-D (178), in addition to releasing VEGF from the ECM (36), indicate the potential importance of this protease for co-ordinating angiogenesis, lymphangiogenesis and fibrinolysis in wound healing.

Hypoxia is a critical stimulus for the angiogenic factors VEGF (95–98) and PlGF (129); however, there is no compelling evidence that it drives the expression of the lymphangiogenic growth factors VEGF-C and VEGF-D. This seems logical given that modulation of tissue oxygenation is not a direct function of the lymphatic vasculature.

The identification of the VEGFs as inducers of angiogenesis and lymphangiogenesis has led to new therapeutic possibilities for diseases associated with vascular function. These include stimulation of these processes using recombinant proteins or gene delivery systems to treat ischemic disease and lymphedema. Additionally, pathological angiogenesis and lymphangiogenesis in solid tumors could be blocked by inhibitors of these growth factors, such as monoclonal antibodies and soluble receptors. However, as our understanding of the mechanisms regulating the VEGFs becomes more extensive there will be opportunities to manipulate the stimuli that influence the production or activity of these growth factors. For example, it may prove clinically attractive to inhibit the proteases that activate the lymphangiogenic growth factors in order to block formation of tumor lymphatics and the resultant lymphogenous metastasis. Likewise, inhibitors of the HIF-1 hypoxia response pathway may be of benefit to block production of VEGF and resultant tumor angiogenesis (99). Future advances in our understanding of the biological regulation of the VEGFs will reveal more potential approaches for manipulating angiogenesis and lymphangiogenesis in the clinic.

This work was supported by the National Health and Medical Research Council of Australia (NH&MRC). BKM is supported by a Melbourne Research Scholarship from the University of Melbourne, SAS by a Senior Research Fellowship from the Pharmacia Foundation, and MGA by a Senior Research Fellowship from the NH&MRC. We thank Tony Burgess for helpful comments.

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